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Application of liquid chromatography with on-line radiochemical detection to metabolism studies on a novel class of analgesics*

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Abstract: Vanilloids are a class of compounds structurally related to capsaicin, the pungent principle of hot peppers, which are under development as a novel class of analgesics. Vanilloids undergo extensive first-pass metabolism when dosed orally to rats and mice. These compounds, as well as capsaicin, would be anticipated to be susceptible to three major routes of metabolism: (ω,β)-oxidation of the alkyl side chain, hydrolysis of the amide bond and conjugation of the phenolic group. Olvanil [*N*-(3-methoxy-4-hydroxybenzyl)oleamide], radiolabelled with either ^{14}C at the benzylic carbon or ^{3}H in the oleyl side chain, was studied in various *in vitro*, *in situ* and *in vivo* metabolism models to determine the major route(s) of intestinal and hepatic metabolism in rats for this new class of compounds. Models used in metabolism studies included isolated hydrolytic enzymes, cell-free intestinal and liver supernatants, hepatocytes, enterocytes, perfused intestine and whole animal studies. Reversed-phase liquid chromatography (LC) with on-line radiochemical detection was used to examine the metabolic profiles from the different models. The major metabolic route for olvanil in both the intestine and the liver was found to be hydrolysis of the amide bond. The benefits of selective ^{14}C and ^{3}H labels in conjunction with LC with on-line radiochemical detection are discussed.

Keywords: *Olvanil; capsaicin; vanilloids; radiochemical detection; hepatocytes; enterocytes; metabolism; reversed-phase liquid chromatography.*

Introduction

Capsaicin is the main pungent principle of hot peppers and has been used as an ingredient in spices all over the world. Capsaicin has been shown to produce antinociception in adult rats after subcutaneous (s.c.) injection [1] and after systemic administration to adult guinea-pigs [2, 3]. The properties of a series of compounds termed vanilloids, structurally related to capsaicin, are being investigated. A prototype vanilloid, olvanil [*N*-(3-methoxy-4-hydroxybenzyl)oleamide], (see Fig. 1) has been shown to be an active antinociceptive and anti-inflammatory agent in rats [4, 5]. Olvanil is active as an antinociceptive agent in rats by both the oral and s.c. routes of administration, with an efficacy better than typical non-steroidal anti-inflammatory drugs [6]. Its anti-nociceptive and anti-inflammatory activities appear to be due to a direct interaction of the drug with C-fibre nociceptors [6].

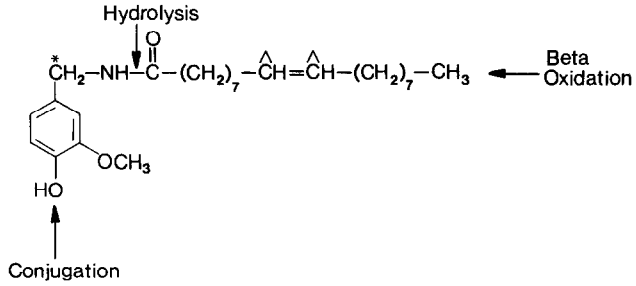

Figure 1
Structure of olvanil and potential routes of metabolism: * denotes the position of the ^{14}C radiolabel, and ∧ denotes the positions of the ^{3}H radiolabel.

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Olvanil has been found to undergo extensive first-pass metabolism following oral dosing in rodents, both mouse [7] and rat (Procter & Gamble, unpublished data). In order to direct synthetic efforts toward the design of more metabolically stable analogues it was deemed necessary to define the major route(s) of metabolism using olvanil as a probe. The structure of olvanil is shown in Fig. 1. Olvanil would be expected to be susceptible to metabolism by either β-oxidation of the fatty side-chain, hydrolysis of the amide bond or conjugation of the phenolic group.

To investigate the potential routes of metabolism, radiolabelled (^{14}C and ^{3}H) olvanil was used in conjunction with a variety of *in vitro*, *in situ* and *in vivo* metabolic models. The metabolism models included isolated hydrolytic enzymes, cell-free rat liver and intestinal supernatants, isolated rat hepatocytes and enterocytes, rat isolated intestinal perfusion and whole animal studies in the rat. In addition to being an economical model, the rat was of particular interest since this species was used extensively in many efficacy models for vanilloids. Reversed-phase liquid chromatography (LC) with sequential UV and on-line radiochemical detection (LC–RAD) was used to examine the metabolic profiles generated in the various metabolic models. Selected ^{14}C and ^{3}H labelled olvanil analogues (see Fig. 1 for positioning of radiolabels) were used to aid in interpreting metabolic profiles.

Experimental

Materials

Chemicals. Olvanil, (9,10-^{3}H)-olvanil (129 Ci g^{-1}), olvanil-*O*-glucuronide and vanillylamine were prepared by Procter & Gamble at Miami Valley Laboratories. (Benzylic-^{14}C)-olvanil (134 μCi mg^{-1}) was prepared by Amersham Corporation. Vanillin and vanillic acid were purchased from Aldrich Chemical Co. Oleic acid was obtained from Nu Check Prep. Porcine liver protease (Type VIII), porcine intestinal peptidase, bovine serum albumin (BSA; RIA Grade) and β-D-glucuronidase (*Escherichia coli*, Type X) were obtained from Sigma Chemical Co.

Animals. Male Sprague–Dawley rats (100–200 g) were obtained from Charles River Laboratories.

Methods

Enzymatic systems. In vitro metabolism of olvanil was examined by mixing a 0.1-ml aliquot of a 5% BSA solution containing ^{14}C-olvanil (20 μCi ml^{-1}, 0.15 mg ml^{-1}) with 0.9 ml of a buffered enzyme solution. The olvanil–enzyme solution was incubated at 37°C in a water bath for 2.5 h. Samples were withdrawn periodically and the enzyme reaction stopped by the addition of 2 vol of acetonitrile. The mixture was vortexed (30 s), centrifuged at 490*g* (10 min) and the supernatant analysed by LC–RAD. The following enzyme solutions were used: porcine intestinal peptidase (1.5 U ml^{-1}; 0.1 M sodium phosphate, pH 7.1) and porcine liver protease (120 U ml^{-1}; 0.1 M phosphate, pH 7.8).

Cell-free liver and intestinal supernatant. The metabolic activities of cell-free liver and intestinal supernatants were examined by incubating 0.1 ml of a 5% BSA solution containing ^{14}C-olvanil (20 μCi ml^{-1}, 0.15 mg ml^{-1}) with 0.9 ml of either supernatant for 1 h in a 37°C water bath. Following incubation, 2 vol of acetonitrile were added to the sample. The resulting solution was vortexed (30 s), centrifuged at 490*g* (10 min) and the supernatant analysed by LC–RAD analysis.

Liver and intestinal cell-free supernatants were prepared by homogenizing rat livers and intestines (both 0.5 g ml^{-1}) in a glass-Teflon Potter–Elvehjem homogenizer with 0.4 M sodium phosphate (pH 7.4). The homogenate was transferred to centrifuge tubes and spun at 9000*g* for 20 min at 4°C. The supernatant was further spun at 105,000*g* for 60 min at 4°C. The supernatant from the second spin was isolated and referred to as a cell-free supernatant.

Isolated hepatocytes and enterocytes. The metabolic activity of isolated hepatocytes and enterocytes was examined by adding a small volume (10 μl) of a mixture of unlabelled olvanil and ^{3}H-olvanil (10.5 mg ml^{-1}, 26.8 mCi g^{-1}) or a mixture of unlabelled olvanil and ^{14}C-olvanil (10.5 mg ml^{-1}, 26.8 mCi g^{-1}) in DMSO to a 4 ml suspension of hepatocytes or enterocytes. This gave an initial olvanil concentration of 63 μM. The cells were incubated in a 37°C water bath with gentle shaking under a 95% O_2:5% CO_2 atmosphere. Aliquots of the suspension were withdrawn periodically and mixed immediately with an equal volume of acetonitrile and vortexed. The supernatant was

analysed by LC–RAD after isolation by centrifugation. The experiment was carried out in triplicate. Hepatocytes and enterocytes were prepared by the methods of Exton [8] and Pinkus [9], respectively.

Isolated intestinal perfusion. Intestinal perfusion was performed with a male rat using a procedure similar to that described by Pang [10]. Briefly, the intestinal blood supply was cut off and the organ perfused by means of a PE-50 cannula inserted into the superior mesenteric artery. The infusion media was a fluorocarbon emulsion (FC-43), 20% (Alpha Therapeutics) continuously aerated with 95% O_2:5% CO_2, pH adjusted to 7.41 and maintained at 37°C. The infusion flow rate was maintained at 5 ml min^{-1} with a MX 2/10 Perfuser (MX International). After a 15-min equilibration period, 0.5 ml of a lecithin-based formulation containing 20 mg of olvanil and 250 μCi of ^{14}C-olvanil was injected intraduodenally. Perfusate effluent was collected from the portal vein, using a PE-10 cannula, over a 60-min time period. The collected perfusate was analysed directly by LC–RAD.

Whole animal studies. Three adult male rats were dosed orally with 200 mg kg^{-1} of ^{14}C-olvanil in an ethyloleate–benzyl alcohol (98:2, v/v) vehicle. Each rat received 500 μCi of ^{14}C-olvanil. Blood was withdrawn 3 h after dosing and the plasma isolated. The plasma was treated with 2 vol of acetonitrile, vortexed and centrifuged at 490*g*. Plasma samples were also treated with β-D-glucuronidase (2500 U ml^{-1}) for 17 h at 23°C and then processed as indicated above. The supernatant was analysed by LC–RAD analysis.

Chromatographic conditions. A Waters Associates C_8-bonded phase Radial Pak column (10 cm × 8 mm, 10 μm) and a Brownlee C_8-bonded phase guard column (3 cm × 4 mm, 7 μm) were used for the chromatographic separation. Mobile phase A was sodium phosphate (containing 0.2% triethylamine; 5 mm pH 3.4 with acetic acid) and mobile phase B was methanol–mobile phase A (90:10, v/v). Samples were injected (0.1–0.5 ml) using a Perkin–Elmer ISS-100 autosampler. The column was eluted at a flow rate of 1.0 ml min^{-1} using a gradient of a 15-min linear ramp from 0 to 100% mobile phase B, followed by a 20-min hold at 100% mobile phase B. The column eluent passed through a Waters Associates Model 450 variable wavelength UV detector set at 280 nm. After leaving the UV detector, the column eluent was mixed in a ratio of 1:3 with Flo-Scint II (Radiomatics Instruments) scintillation cocktail. The combined eluent was passed through the 0.5-ml flow cell of a Flo-One Beta IC/DS on-line radiochemical detector (Radiomatics Instruments).

Results

Isolated enzymes

The LC–RAD profiles for the incubation of ^{14}C-olvanil with porcine liver protease (Type VIII) and porcine intestinal peptidase showed that parent drug was degraded to give a component coeluting with vanillylamine (data not shown). Olvanil was not metabolized by a number of other hydrolytic enzymes: (α-chymotrypsin, elastase, papain, cathepsin C, pepsin, leucine aminopeptidase or porcine liver esterase).

Cell-free liver and intestinal supernatants

Incubation of ^{14}C-olvanil with cell-free liver supernatant resulted in the generation of vanillylamine as the major radioactivity peak (data not shown). This was confirmed by spiking the sample with vanillylamine. Cell-free intestinal supernatants were also found to hydrolyse ^{14}C-olvanil (data not shown).

Isolated enterocytes

Olvanil was metabolized extensively by enterocytes under the conditions described in Methods (Table 1). Isolated enterocytes were found to metabolize ^{14}C-olvanil to several major components (Fig. 2A). Spiking experiments with vanillylamine and vanillin demonstrated that two of the major metabolites coeluted with these standards. The identity of

Table 1
^{14}C-Olvanil incubation with rat enterocytes and rat hepatocytes

Incubation time (min)	Percent olvanil remaining* Enterocytes	Hepatocytes
5	95 ± 1	69 ± 3
15	75 ± 2	21 ± 5
30	46 ± 4	4 ± 2
60	1 ± 0.2	ND

*Mean ± SD of three samples from one rat.
ND = none detected.

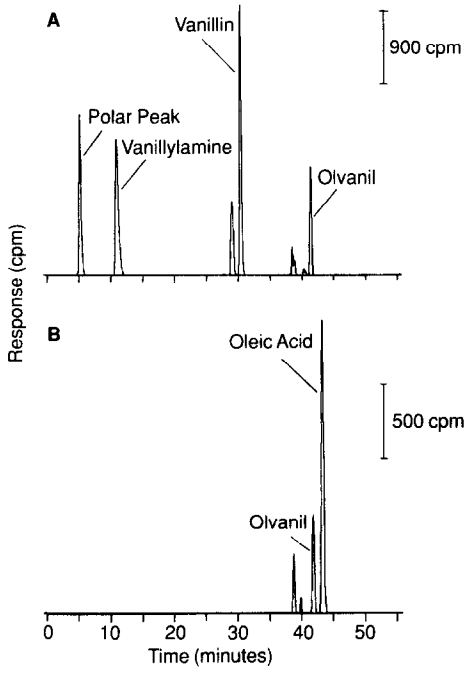


Figure 2
Representative LC–RAD chromatograms following 60-min incubations of olvanil with enterocytes: A, ^{14}C-olvanil; and B, ^{3}H-olvanil.

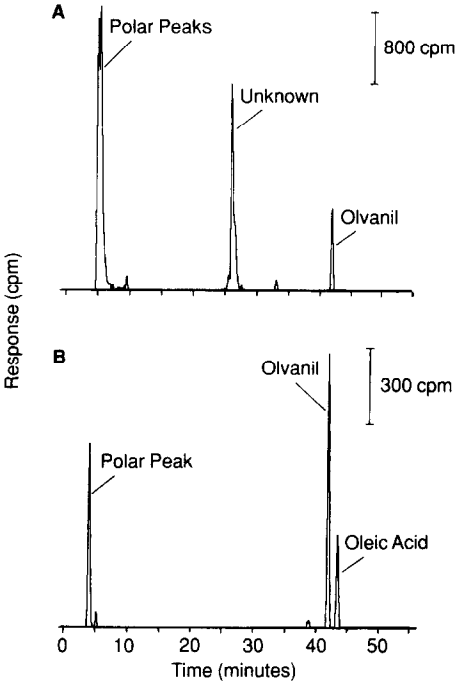


Figure 3
Representative LC–RAD chromatograms following incubation of olvanil with rat hepatocytes: A, ^{14}C-olvanil, 30 min incubation; and B, ^{3}H-olvanil, 5 min incubation.

these two components was further confirmed by incubating vanillylamine with isolated enterocyte supernatant and analysing the supernatant by LC with UV detection. The vanillylamine was completely metabolized by the enterocyte supernatant to give a metabolite coeluting with vanillin.

The enterocyte supernatant was obtained by removing the enterocytes by centrifugation, suggesting the enzymes involved are released or leak from the enterocytes. Incubation of ^{3}H-olvanil with isolated enterocytes generated one major metabolite (Fig. 2B). This metabolite accounted for an average of 74% of all metabolites formed over the time course of the incubation and was shown to coelute with oleic acid.

Isolated hepatocytes

Incubation of ^{14}C-olvanil with isolated hepatocytes also resulted in extensive metabolism of olvanil (Table 1). A major polar metabolite peak, eluting near the void volume, and a second, less polar metabolite were observed (Fig. 3A). Free vanillylamine was not found in hepatocyte incubations with olvanil; however, conjugated vanillylamine could have been a component of the polar peaks. The metabolism of ^{3}H-olvanil was also investigated using isolated hepatocytes. Hepatocytes were found to generate two major components from ^{3}H-olvanil: a polar metabolite peak eluting near the void volume and a component coeluting with oleic acid (Fig. 3B). The oleic acid peak accounted for an average of 24% of the metabolites formed over the time course of the incubation.

Isolated perfused intestine

Perfusate collected via the portal cannula following the introduction of ^{14}C-olvanil into the duodenum was found to contain three major components: a polar metabolite, a metabolite coeluting with vanillylamine and unmetabolized olvanil (see Fig. 4). Based on total ^{14}C activity associated with the metabolites, it was estimated olvanil was 90% metabolized upon absorption across the intestinal membranes.

Whole animal studies

An LC–RAD chromatogram for the injection of rat plasma obtained 3 h following oral dosing with ^{14}C-olvanil is shown in Fig. 5A. A polar peak eluting near the void volume and a non-polar peak coeluting with olvanil-*O*-glu-

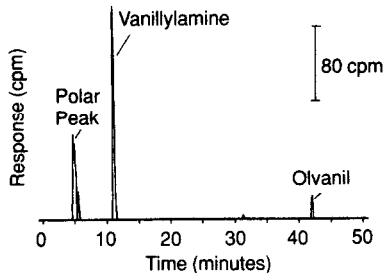


Figure 4
An LC–RAD chromatogram of a 45-min portal canulla perfusate obtained from the isolated rat intestinal perfusion model.

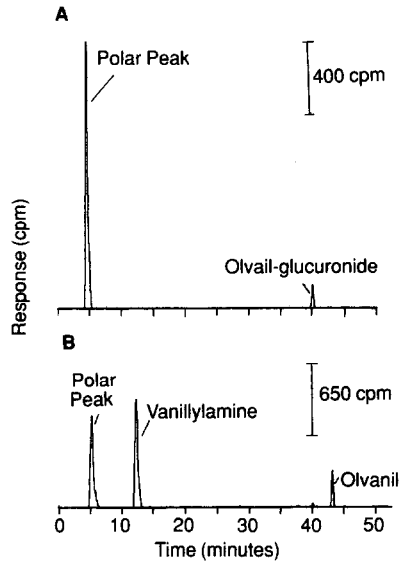


Figure 5
Representative LC–RAD chromatograms of: A, a plasma sample obtained from a rat 3 h after an oral dose of ^{14}C-olvanil; and B, the same plasma sample after treatment with β-D-glucuronidase.

curonide were observed. Treatment of the plasma with β-D-glucuronidase resulted in the cleavage of both of the metabolite peaks and the appearance of two new metabolites (Fig. 5B). One of the new metabolite peaks coeluted with vanillylamine and the other metabolite coeluted with olvanil.

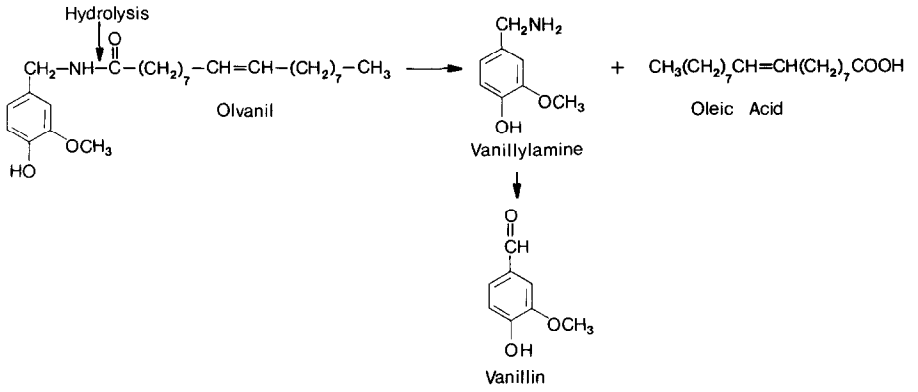
Discussion

Olvanil undergoes an extensive first-pass metabolism in rats and mice with an estimated oral bioavailability of ≤1%. Understanding the major route(s) of vanilloid metabolism could lead to the design of metabolically stable analogues. The olvanil structure is potentially susceptible to hydrolysis, β-oxidation and conjugation (see Fig. 1). The metabolism of structurally related capsaicin has been studied by a number of investigators. Lee and Kumar [11] and Miller *et al.* [12] reported capsaicin to be metabolized via ring hydroxylation at the 5-position in rat liver homogenates. Kawada *et al.* demonstrated that the metabolism of dihydrocapsaicin, both *in vivo* and *in vitro* (cell-free extracts of jejunal tissue), involved hydrolysis of the amide bond [13]. A potential hydrolytic and glucuronidation route of capsaicin metabolism was demonstrated by Leelahuta *et al.* using rat, mouse and hamster cell-free liver supernatants [14].

Initial studies with olvanil described here involved a series of *in vitro* metabolism studies. These studies progressed from isolated enzyme systems to isolated enterocytes and hepatocytes, systems more representative of the intact animal. In these studies, radiolabelled olvanil (^{13}C or ^{3}H) was used, and metabolism monitored by LC–RAD analysis. The incubation of olvanil with isolated hydrolytic enzyme systems (porcine intestinal peptidase and porcine liver protease) resulted in the cleavage of the amide bond, supported by the generation of vanillylamine (see Scheme 1). The use of cell-free intestinal and liver supernatants demonstrated that rat intestine and liver possess enzyme(s) capable of hydrolysing the olvanil amide bond, again evidenced by the generation of vanillylamine in this model system.

Olvanil was metabolized extensively under the conditions used for the *in vitro* isolated enterocyte incubation (Table 1). Isolated enterocyte incubations with ^{14}C-olvanil resulted in the generation of vanillylamine and vanillin, a metabolic product of vanillylamine, as the major metabolic products (Fig. 2A and Scheme 1). The presence of hydrolysis products does not provide proof of hydrolysis as the first metabolic reaction of the parent drug in enterocytes. An alternative metabolic reaction, such as β-oxidation, could occur first and the resulting metabolite undergo rapid hydrolysis to generate vanillylamine. However, the generation of large amounts of ^{3}H-oleic acid (74% of total metabolites) from the incubation of enterocytes with ^{3}H-olvanil demonstrated that hydrolysis was the first and dominant route of olvanil metabolism for enterocytes (Fig. 2B; Scheme 1).

Incubation of ^{14}C-olvanil with hepatocytes did not provide a clear indication that hydrolysis had occurred (Fig. 3A). None of the



Scheme 1

metabolites produced coeluted with any of the available hydrolysis byproduct standards: vanillylamine, vanillin or vanillic acid. However, incubation of ^{3}H-olvanil with hepatocytes resulted in the formation of a significant amount (24% of total metabolites) of free ^{3}H-oleic acid (Fig. 3B). The selective positioning of the ^{3}H-radiolabel on the side-chain of olvanil allowed a rapid and definitive interpretation of hepatic hydrolysis activity not afforded by the ^{14}C-olvanil. Consistent with the cell-free liver supernatant work, this demonstrated hydrolysis was also a significant route of hepatic metabolism.

In vitro experiments pointed to hydrolysis as the major route of olvanil metabolism in the rat intestine and liver. The next step taken was to examine the metabolism of olvanil in an *in situ* model, the perfused rat intestine. The perfusate from a portal cannula was collected following intraduodenal injection of ^{14}C-olvanil into a cannulated rat. LC–RAD analysis of perfusate showed vanillylamine and a polar metabolite to be the major intestinal tissue metabolites (Fig. 4). Olvanil was 90% metabolized on passage through the intestinal tissue. The *in situ* results are consistent with the *in vitro* results and strengthen the case that hydrolysis is the major route of vanilloid metabolism by rat intestinal tissue. The identity of the polar component shown in Fig. 4 was not pursued.

The final step taken in studying olvanil metabolism was to examine the *in vivo* handling of the drug. Plasma samples were obtained from rats 3 h after oral dosing with ^{14}C-olvanil, this time corresponding to the C_{max} for the parent drug in plasma. LC–RAD profiles revealed two major metabolic components: a polar metabolite eluting near the void volume and a component coeluting with authentic olvanil-*O*-glucuronide (Fig. 5A). The polar component accounted for the majority (80–90%) of the ^{14}C activity in the rat plasma samples examined (n = 3). Treatment of the plasma sample with β-D-glucuronidase resulted in the partial elimination of the polar component and the complete elimination of the olvanil-*O*-glucuronide peak. Two new components appeared in the chromatogram. One of the new components coeluted with vanillylamine and the other new component coeluted with olvanil (Fig. 5B). The *in vivo* plasma results are consistent with the *in vitro* and *in situ* findings and support the conclusion that hydrolysis is a major route of vanilloid metabolism. The *in vivo* work indicated that glucuronidation of the phenolic group may also be an important route of olvanil metabolism.

Conclusions

The use of radiolabelled olvanil in various *in vitro*, *in situ* and *in vivo* models showed hydrolysis to be the dominant route of olvanil metabolism in the rat intestine and liver. The use of LC with on-line radiochemical detection in conjunction with ^{14}C- and ^{3}H-olvanil provided a rapid means of comparing metabolism in the various model systems. The results of the *in vitro*, *in situ* and *in vivo* olvanil metabolism work have been used to design metabolically more stable vanilloids. The effect of structural modifications on vanilloid metabolism will be described elsewhere.

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